Neuroinflammation and α-Synuclein Dysfunction Potentiate Each Other Driving Chronic Progression of Parkinson's Disease

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Supplemental Methods

Immmunohistochemistry, confocal double-label immunofluorescence, and cell counting

Immunostaining and double-label immunofluorescence were performed as described previously using the following primary antibodies: SYN211 (1:500; Millipore), nSYN514 (1:150; Santa Cruz), and antibodies against a neuron-specific nuclear protein (Neu-N; 1:2000; Chemicon), tyrosine hydroxylase (TH; 1:1000; Sigma), or ionized calcium-binding adaptor molecule 1 (Iba-1; 1:500; Wako Chemicals). Briefly, the 30 μm paraformaldehyde-fixed floating brain sections were blocked with appropriate normal serum followed by incubation overnight at 4°C with primary antibodies. The bound primary antibodies were visualized by incubation with an appropriate biotinylated secondary antibody, followed by the Vectastain ABC reagents and color development with 3,3′-diaminobenzidine (DAB).

For double-label immunohistochemical staining, the 30 μm frozen brain sections were incubated with 99% formic acid for 5 min for antigen retrieval, followed by blocking with 5% horse serum/1% BSA. These brain sections were first stained with the anti-Neu-N antibody using DAB as a chromogen and nickel sulfate as an intensifying agent (dark blue) followed with nSYN514 antibody (specific for nitrated human α-syn) using DAB as a chromogen (brown). Images were recorded with a CCD camera and the MetaMorph software (Gao et al. 2003; Giasson et al. 2000).

Double-label immunofluorescence was performed by staining brain sections with antibodies against Neu-N and TH, anti-TH antibody combined with SNY211, or anti-Iba1 antibody combined with anti-TH antibody, followed by incubation with Alexa-488 (green) and Alexa-594 (red) conjugated secondary antibodies (1:1000). After mounting the sections onto glass slides with the prolong antifade reagents, fluorescent images were obtained with a Zeiss LSM 510 NLO laser scanning confocal microscope fitted with an Argon ion laser (488 nm) and a HeNe laser (543 nm) and recorded with the Zeiss LSM510 software.

To monitor dopaminergic neurodegeneration, the number of nigral TH-immunoreactive (TH-IR) neurons was counted. To ensure the accuracy of the count, a normal distribution (rostral to caudal) of SN TH-positive neurons was first established as described previously (Zhang et al. 2004). No apparent differences were observed in the distribution patterns between wildtype and transgenic mice. Six evenly spaced brain sections (every fourth section from the rostral of a series of 24 sections that cover the entire extent of SN) were counted by two individuals blind to the treatment. Brain sections from 3-6 mice in each treatment group were used for the visual enumeration (Zhang et al. 2004).

Sequential biochemical fractionation

Sequential protein extraction was utilized to isolate and detect insoluble α-syn based on the solubility of proteins for the initial removal of highly soluble proteins. Proteins from the insoluble pellets were then extracted using buffers with increasing solubilization strength. Briefly, midbrain tissue samples were homogenized in 3 ml/g of high-salt (HS) buffer (50 mM Tris-HCl [pH 7.5], 10 mM EGTA, 5 mM MgSO4, 750 mM NaCl, 20 mM NaF and protease inhibitor cocktail) and centrifuged at 100,000 × g for 30 min at 4°C. Pellets were re-extracted with HS buffer. The combined supernatant was used as the HS fraction. The pellets were sequentially extracted with HST buffer (HS buffer containing 1% Triton X-100). After centrifuge at 100,000 × g for 30 min at 4°C, the pellets were dissolved in raidioimmunoprecipitation assay (RIPA) buffer containing 50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 5 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate and 0.1% SDS, followed by sedimentation at 100,000 × g for 60 min at 4°C. The HS-, HST- and RIPA-soluble supernatant were used as HS fraction, HST fraction, and RIPA fraction, respectively. The RIPA-insoluble pellets were then re-extracted in 2% SDS buffer by sonication and heated to 100°C for 10 min (Gao et al. 2008; Giasson et al. 2002).

Gel electrophoresis and western blotting analysis

In all biochemistry studies except sequential biochemical fractionation analysis, the 2% SDS buffer (2% SDS in 50 mM Tris-HCl [pH 7.5]) was used to extract both soluble and aggregated proteins (e.g. α-synuclein) from stratum, SN, or whole brains. Protein samples were separated on 4-12% SDS-PAGE gels. The distribution of α-syn and other proteins was determined by western blotting using SYN211 (1:1000), nSYN514 (1:250), α-synuclein (C-20)-R (reactive to mouse, rat and human α-syn), or antibodies specific for inducible nitric oxide synthase (iNOS; 1:500), macrophage antigen complex 1 (Mac1; 1:1000), TH (1:5000), Neu-N (1:500), cyclooxygenase 2 (COX2; 1:1000), or gp91^{phox} (1:2000). A monoclonal anti-β-actin antibody (1:5000) was included as an internal standard to monitor loading errors (Gao et al. 2008; Giasson et al. 2002).

Tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) assays

The levels of serum TNF-α and IL-1β were measured with commercial enzyme-

linked immunosorbent assay kits from R&D Systems.

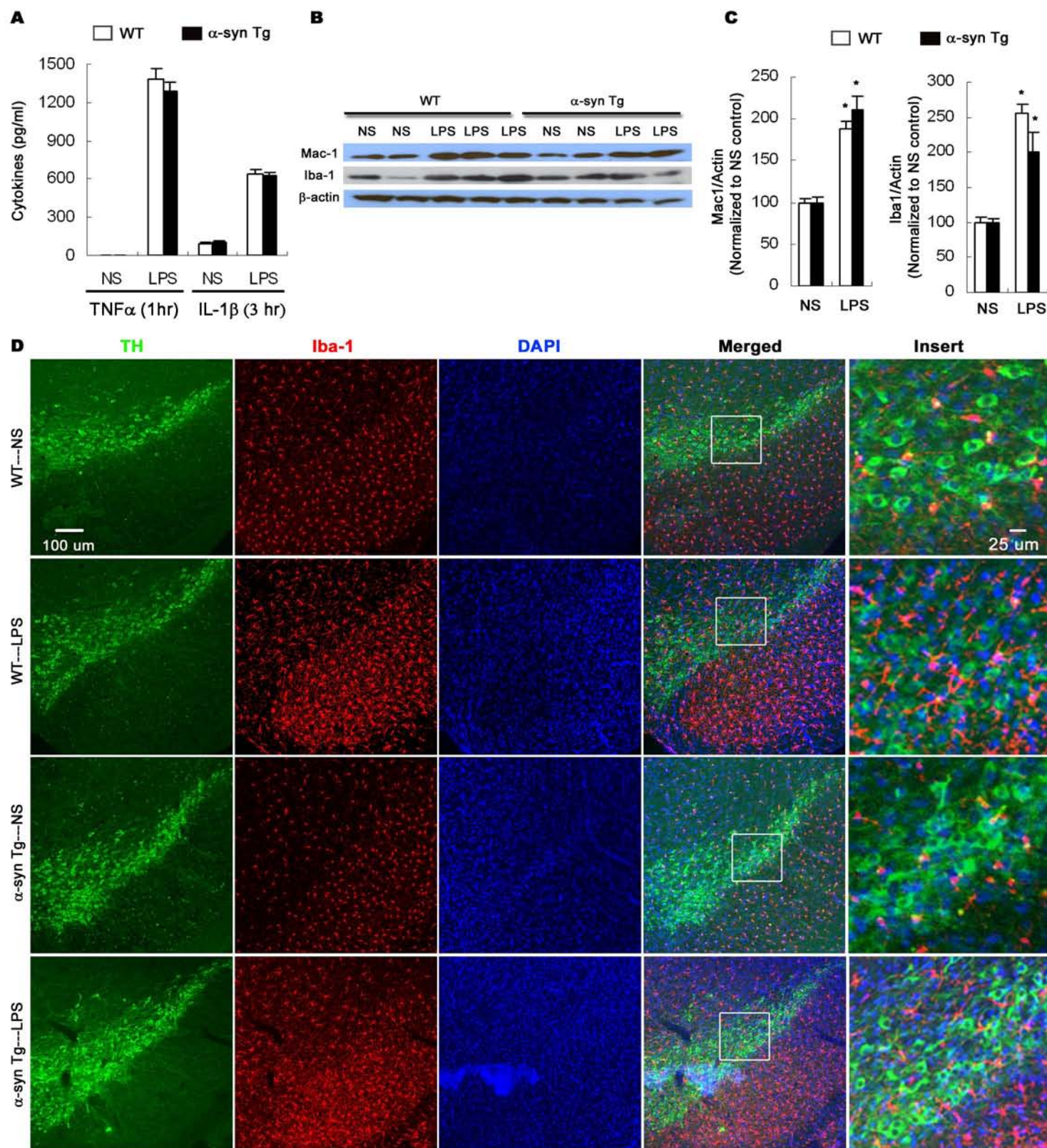**Densitometry analysis of TH-IR fibers in the stratum**

Image J software was utilized to measure and to compare the optical intensity of TH-IR fibers in the stratum regions. Eight evenly spaced brain sections from a series of 64 sections that cover the entire stratum were used for the densitometry analysis. We randomly drew 10 same-sized ROI (region of interest) in each stratum image of every brain section. We measured and averaged the mean intensity of all ROI to generate the mean intensity of each striatal images. The mean intensity of eight TH-IR striatal images from each treated mouse was analyzed and then averaged within each treatment group. The mean densitometry value of TH-IR fibers of each treatment group was normalized to NS-injected WT control.

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Supplemental Material, Figure 1. Similar acute systemic and brain inflammatory reactions in wildtype and α-syn Tg mice after LPS challenge

The intraperitoneal injection of LPS (3 X 10^6 EU/kg) caused similar acute systemic inflammatory reactions in wildtype (WT) and Tg mice, as evidenced by similar levels of serum TNF-α and IL-1β in WT and Tg mice. **(B, C)** Western blotting showed increased expression of Mac1 and Iba-1 in mouse brains 1 day after LPS injection. There was no significant difference in the levels of either Mac1 or Iba-1 between the two genotypes. *p<0.05 compared with the corresponding NS-injected control. (**D**) Double-label immunofluorescence revealed activation of microglia in the mouse SN one day after LPS injection. Nigral microglia exhibited the typical ramified morphology of resting microglia in NS-injected mice. Microglia of LPS-injected mice were activated with the characteristics of larger cell bodies, thicker processes, and intensified Iba-1 staining. These morphologic alterations in WT and Tg mice were indistinguishable between genotypes. Three mice from each group were used for cytokine measurement, western blotting and immunstaining. Scale bars: A, 100um; insert, 25um.